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(71) Applicant: UNIVERSITY OF UTAH RESEARCH FOUNDA-TION [US/US]; 210 Park Building, Salt Lake City, UT 84112 (US).

(72) Inventors: JACOBS, Harvey; 22 Richard Drive, Dallas, PA 18614 (US). KIM, Sung, Wan; 1711 Devonshire Drive, Salt Lake City, UT 84108 (US). MENARD, Virginie; 130 S. 1300 E. #503, Salt Lake City, UT 84102 (US).

(74) Agents: HOWARTH, Alan, J. et al.; Thorpe, North & Western, L.L.P., Suite 200, 9035 South 700 East, Sandy, UT 84070 (US).

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(57) Abstract

A method for delaying onset of insulin dependent diabetes mellitus (IDDM) in an individual predisposed to developing the disease is disclosed. The method comprises administering a composition comprising an immunologically effective monoclonal antibody or fragment thereof against glutamic acid decarboxylase (GAD) coupled to a nonimmunogenic hydrophilic polymer that provides a hydration shell around the monoclonal antibody for inhibiting immune recognition thereof. Poly(ethylene glycol) is a preferred polymer. A method of reducing insulitis in an IDDM patient and a composition therefor are also described.

"TARGETING OF CONJUGATES OF POLY(ETHYLENE GLYCOL) AND ANTIBODIES AGAINST GLUTAMIC ACID DECARBOXYLASE TO ISLET CELLS"

CROSS-REFERENCE TO RELATED APPLICATIONS

5   This application claims the benefit of U.S. Provisional Application No. 60/004,109, filed September 21, 1995.

BACKGROUND OF THE INVENTION

       This invention relates to a method of treating
10   insulin dependent diabetes mellitus (IDDM). More particularly, the invention relates to a method of delaying onset of IDDM by systemically administering a composition comprising a modified anti-glutamic-acid-decarboxylase antibody to an individual in need thereof.
15   The pancreas is composed of two major tissues, the acini, which secrete digestive juices into the duodenum, and the islet cells of Langerhans, which secrete insulin and glucagon into the blood. Insulin functions in the storage of excess energy sources. For example, insulin
20   stimulates (a) the storage of carbohydrates in the muscles and in the liver as glycogen, (b) the storage of fat in adipose tissue, and (c) the uptake and storage of amino acids as proteins in cells. Insulin's main role, however, is in the homeostatic control of blood glucose
25   through facilitated glucose uptake into muscle cells. At a normal blood glucose level of about 100 mg/dl, the rate of insulin secretion is about 25 ng/min/kg. When glucose levels rise after a meal, glucose stimulates beta cell receptors to secrete insulin, resulting in a
30   10-25-fold increase over the basal rate. Once blood glucose levels decrease, the beta cells rapidly turn off insulin production and return to basal levels.
       Insulin dependent diabetes mellitus (Type I, IDDM) results from diminished secretion of insulin by the beta
35   cells. Diabetes is a process involving different stages of disease progression: obligatory genetic predisposition, followed by a precipitating event - either a genetic mutation or an environmental insult, all leading to autoimmune destruction of islet/beta

cells. It has been noted that up to several years prior to onset of diabetes, greater than 70% of pre-clinical IDDM patients exhibit circulating antibodies directed against islet/beta cell antigens. E.g. I. Deschamps, Life Table Analysis of the Risk of Type 1 (Insulin-dependent) Diabetes Mellitus in Siblings According to Islet Cell Antibodies and HLA Markers, 35 Diabetologia 951-57 (1992).

IDDM is characterized by cellular immunological destruction of beta cells, resulting in a lack of insulin secretion and leading to the severe pathogenesis of the disease. E.g., L.C. Harrison et al., Islet-reactive T Cells Are a Marker of Preclinical Insulin-dependent Diabetes, 89 J. Clin. Invest. 1161-65 (1992). Different parameters including genetic predisposition, e.g., I. Deschamps, 35 Diabetologia 951-57, supra, intervenous glucose tolerance (IVGT), and the presence of antibodies, T.J. Wilkin et al., Autoimmune Diabetes and the Germ Theory of Disease, 35 Diabetologia 187-89 (1992), have been shown as causative agents in the development of diabetes.

The contribution of antibodies in the development of diabetes, as an autoimmune disease, has been demonstrated by many researchers. Several preclinical antibody markers have been isolated, identified, and correlated with IDDM including antibodies against insulin, insulin receptors, islet cell surface antigens, and carboxypeptidase H. Statistical studies, e.g., U. Roll et al., Associations of Anti-GAD Antibodies with Islet Cell Antibodies and Insulin Autoantibodies in First-degree Relatives of Type I Diabetes Patients, 43 Diabetes 154-60 (1994), show that the pattern of antibody expression, especially involving insulin antibodies (IAA) and islet cell antibodies (ICA) is highly correlated to the onset of diabetes. Patients exhibiting antibodies to both IAA and ICA have a greater

than 90% probability of devloping diabetes within 6 years.

Of particular importance in the causative nature of pre-diabetic antibodies is the identification of a subset of ICA directed against glutamic acid decarboxylase (GAD). Anti-GAD antibodies have been shown to be present in about 70% of relatives demonstrating ICA and IAA antibody titers. E.g., U. Roll et al., supra. Of this population, about 66% of the patients still develop diabetes. Furthermore, studies have demonstrated that the immune system of young mice can develop tolerance to anti-GAD antibodies through the continued injection of GAD and not develop IDDM. P.Z. Zimmet et al., Latent Autoimmune Diabetes Mellitus in Adults (LADA): the Role of Antibodies to Glutamic Acid Decarboxylase in Diagnosis and Prediction of Insulin Dependency, 11 Diabetic Medicine 299-303 (1994). In this process, the enzyme GAD is not recognized as a foreign protein. Thus, autoimmune recognition does not occur and there is no destruction of beta cells and normoglycemia is exhibited.

The initial step in an autoimmune response is the nonspecific recognition, phagocytosis, and processing of proteins or other molecules as foreign antigens by antigen-presenting cells (APC's): Langerhans cells (skin), dendritic cells (spleen, lymph nodes), and monocytes (blood). After phagocytosis, the partially degraded antigen fragment is transferred to a cell-surface glycoprotein known as the major histocompatibility complex class II (MHC II). B cells also assimilate and process specific antigens. Antigens bind to antibodies on the B cell surface, become assimilated, processed, and expressed by MHC II proteins, similar to APC's. The processed antigen is recognized by helper T cells through their CD4 T cell receptor. The recognition, interaction, and proliferation of B cells through helper T cell contact

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is essential for a strong immune response, since B cells are the chief antibody secreting cells. The B memory cells lack soluble antibody, yet they retain surface-bound specific antigen receptors. These long-lived

5   cells are primed for response to subsequent antigenic exposures. D.P. Stites et al., Basic and Clinical Immunology (1987); E. Harlow & D. Lane, Antibodies: A Laboratory Manual (1988).

CD8 cytotoxic T cell receptors recognize both

10   antigenic epitopes and helper T cell surface receptors. E.g., B.J. Bradley et al., CD8 T Cells Are Not Required for Islet Destruction Induced by a CD4 Islet-Specific T-Cell Clone, 41 Diabetes 1603-08 (1992). It is the infiltration, interaction, fusion, and lysis of the

15   antigen effected cell with the cytotoxic T cell that leads to cell destruction. In addition, destruction of antigen affected cells can occur through a humoral immunity process. Briefly, IgG antibodies bound to antigens will activate the complement fixing pathway

20   through their Fc fragment, thereby leading to cell death. D.P. Stites et al., Basic and Clinical Immunology (1987).

Circulating antibodies against islet/beta cell antigen (islet cell antibodies (ICA)) were first

25   observed by G.F. Botazzo et al., Islet Cell Antibodies in Diabetes Mellitus with Autoimmune Polyendocrine Deficiency, Lancet 1279-83 (1974). The specific identification and cellular location of the relevant antigens remains unknown. Initial studies proposed that

30   the ICA target (antigen) had the biochemical characteristics of a gangliocide. F. Dotta et al., Pancreatis Islet Ganglioside Expression in Non-obese Diabetic Mice; Comparison with C57B1/10 Mice and Changes After Autoimmune B-cell Desctruction, 130 Endocrinology

35   37-42 (1992). ICA can be visualized by immunofluorescence; the serum of a newly diagnosed IDDM patient is incubated with frozen diabetic pancreas

cells, and then islet ICA can be visualized using
fluorescent labeled second antibodies.   E.g., C.J.
Greenbaum et al., Improved Specificity of ICA Assays in
the Fourth International Immunology of Diabetes Serum
5   Exchange Workshop, 41 Diabetes 1570-74 (1992); C.J.
Greenbaum et al., Fifth Inernational Serum Exchange
Workshop for Insulin Autoantibody (IAA) Standardization,
35 Diabetologia 798-800 (1992). This method identifies
ICA as a group of antibodies, and does not distinguish
10   between islet or beta cell specificity. As a group of
antibodies, ICA antibodies have been detected at very
low levels in the average population, M. Landin-Olsson
et al., Predictive Value of Islet Cell and Insulin
Autoantibodies for Type 1 (Insulin-dependent) Diabetes
15   Mellitus in a Population-based Study of Newly-diagnosed
Diabetic and Matched Control Children, 35 Diabetologia
1068-73 (1992); C. Levy-Marshal et al., Islet Cell
Antibodies in Normal French School Children, 35
Diabetologia 577-82 (1992), yet high titers predict a
20   high probability of developing IDDM. E.g., A. Gottsater
et al., Islet Cell Antibodies and Fasting Plasma C-
peptide During the First 10 Years After Diagnosis in
Patients with Diabetes Mellitus Diagnosed in Adult Age,
5 Diab. Nutr. Metab. 243-48 (1992).   Furthermore,
25   association of ICA with a genetic characteristic of
major histocompatibility complex (MHC:DR3+DR4+) results
in an 84% probability of developing IDDM. I. Deschamps,
35 Diabetologia 951-57, supra.
          Recent research has identified antibodies directed
30   against a beta cell antigen known as glutamic acid
decarboxylase (GAD).   E.g., M.R. Christie et al.,
Binding of Antibodies in Sera from Type 1 Diabetic
Patients to Glutamate Decarboxylase from Rat Tissues--
Evidence for Antigenic and Non-antigenic Forms of the
35   Enzyme, 35 Diabetologia 380-84 (1992); M.R. Christie, et
al., Antibodies to GAD and Tryptic Fragments of Islet
64K Antigen as Distinct Markers for Development of IDDM-

-Studies with Identical Twins, 41 Diabetes 782-87 (1992). GAD is an enzyme associated with the generation of the neurotransmitter, GABA. H.J. DeAizpurua & L.C. Harrison, Glutamic Acid Decarboxylase in Insulin Dependent Diabetes Mellitus, 8 Diabetes/Metabolism Reviews 133-47 (1992). GAD has a molecular weight of about 64,000, and has been shown to exist in different isomeric forms, such as GAD-65 and GAD-67. Circulating antibodies directed against a GAD-67 epitope have also been discovered in Stiffman Syndrome. E. Bjork et al., GAD Autoantibodies in IDDM, Stiff-Man Syndrome, and Autoimmune Polyendocrine Syndrome Type I Recognize Different Epitopes, 43 Diabetes 161-65 (1994).

In the case of IDDM, anti-GAD antibodies are becoming recognized as significant predictors for the development of diabetes; 60-70% of the patients who present anti-GAD antibodies will develop diabetes. E.g., C.H. Thivolet et al., Glutamic Acid Decarboxylase (GAD) Autoantibodies are Additional Predictive Markers of Type 1 (Insulin-dependent) Diabetes Mellitus in High Risk Individuals, 35 Diabetologia 570-76 (1992). Like ICA, the presence of anti-GAD antibodies is associated with genetic MHC characteristics, thereby increasing the probability of IDDM. S.W. Serjeantson et al., Antibodies to Glutamic Acid Decarboxylase Are Associated with HLA-DR Genotypes in both Australians and Asians with Type 1 (Insulin-dependent) Diabetes Mellitus, 35 Diabetologia 996-1001 (1992). Others have shown that the presence of anti-GAD antibodies correlates with T cell activation and the development of insulitis. R. Tisch et al., Immune Response to Glutamic Acid Decarboxylase Correlates with Insulitis in Non-obese Diabetic Mice, 366 Nature 72-75 (1993). In yet another pertinent study, GAD was injected into young (prediabetic) NOD mice and GAD was found to block the development of T cell reactivity toward beta cell antigens, thereby preventing insulitis and diabetes.

D.L. Kaufman et al., Spontaneous Loss of T-Cell Tolerance to Glutamic Acid Decarboxylase in Murine Insulin-dependent Diabetes, 344 Nature 69-72 (1993).

5   Other types of antibodies have been found in the serum of prediabetic and diabetic patients. The antibodies with the greatest signficance for diagnosis are the ICA antibodies, but many other types have been studied, although their importance has yet to be determined. The finding of insulin autoantibodies in
10 the serum of prediabetic patients, prior to insulin therapy, correlates with the rate of progression of diabetes. As with the ICA antibodies, international workshops have resulted in standardization of IAA titration. C.J. Greenbaum et al., 35 Diabetologia 798-
15 800, supra. On the other hand, it is possible to induce diabetes in NOD mice by transfection of specific genes related to the insulin receptor. E.g., M.A. Lipes et al., Progression to Diabetes in Non-obese Diabetic (NOD) Mice with Transgenic T Cell Receptors, 259 Science 1165-
20 69 (1993). These antibodies have been reported in some studies, but their role and importance have not been clarified. E.g., G.S. Eisenbarth et al., Pathogenesis of Insulin-dependent (Type I) Diabetes Mellitus, in C.R. Kohn & G. Weiz eds., Joslin's Diabetes Mellitus 216-39
25 (13th ed., 1994). Some studies have investigated the role of islet cells surface antibodies, but the difficulty in measuring these antibodies and the lack of real evidence of their presence in prediabetic pateints make this a difficult tool to estimate the onset of
30 diabetes. E.g, M. Vives et al., Reevaluation of Autoantibodies to Islet Cell Membrane in IDDM--Failure to Detect Islet Cell Surface Antibodies Using Human Islet Cells as Substrate, 41 Diabetes 1624-31 (1992). Finally, antibodies to carboxypeptidase H (CPH) have
35 been found in 30% of prediabetic patients who will develop diabetes. G.S. Eisenbarth et al., in Joslin's Diabetes Mellitus 216-39, supra. These statistics and

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current knowledge of this CPH antibody allow the evaluation of these autoantibodies as markers compared to anti-GAD and ICA antibodies.

5   The only currently approved treatment for IDDM is the daily administration of exogenous insulin. E.g., R.B. Tattersall & E. Gale, Patient Self-monitoring of Blood Glucose and Refinements of Coventional Insulin Treatment, 70 Am. J. Med. 177-80 (1981). Other experimental protocols include computer controlled
10   infusion pumps to deliver insulin in relation to blood glucose, e.g., F.J. Fogt et al., Development and Evaluation of a Glucose Analyzer for a Glucose-Controlled Insulin Infusion System (Biostator), 24 Clin. Chem. 1366-81 (1978), bio-feedback mechanisms with
15   concanavalin A-glycoylated insulin, e.g. S.W. Kim et al., A self-regulating Insulin Delivery System, Excerpta Medica 25-32 (1990), and insulin delivery devices utilizing glucose oxidase, K. Ishihara et al., Glucose Induced Permeation Through a Complex Membrane Consisting
20   of Immobilized Glucose Oxidase and a Poly(amine), 16 Polymer J. 625-42 (1984). In these treatments, exogenous insulin must be administered since all beta cell function has been previously destroyed. Another approach to treating IDDM is to transplant functioning
25   islet/beta cells into the diabetic patient. E.g., E. Reich et al., Prevention of Diabetes in NOD Mice by Injection of Autoreactive T-lymphocytes, 38 Diabetes 1647-51 (1989). Theoretically, this approach will provide euglycemia for the patient. However, the islet
30   cells are usually from a different species, and transplant-related rejection is often observed. E.g., R.P. Lanza et al., Xenotransplantation of Canine, Bovine and Porcine Islets in Diabetic Rats without Immunosuppresion, 88 Proc. Nat'l Acad. Sci. USA 11100-03
35   (1991).

The A different approach is to prevent or delay the development of diabetes. In some studies,

immunosuppressant therapy was initiated after IDDM
markers (genetic, ICA, IAA, GAD) were detected. E.g.,
P.J. Bingley et al., Can We Really Predict IDDM?, 42
Diabetes 213-20 (1993). Immunosuppressives, such as
5   corticosteroids, azathioprine, and cyclosporin A have
been used to treat diabetes. E.g., A. Lernmark, Immune
Intervention Yes, but for What Reason, for Whom, When
and How?, 35 Diabetologia 1096-98 (1992). If
administered prior to total beta cell destruction, these
10   agents specifically blocked cellular immune activity.
The beta cells were protected from further destruction,
allowing regeneration of viable cells, and transient
return to euglycemia. E.g., H. Kolb et al.,
Immunomodulatory Drugs in Type I Diabetes, in G.S.
15   Eisenbarth ed., Immunotherapy of Diabetes and Selected
Autoimmune Diseases 111-23 (1989). However, the side
effects of these drugs, especially in diabetic children,
outweighed their usefulness. A. Lernmark, 35
Diabetologia 1096-98, supra.
20   From an immunotherapeutic approach, overt early
stage diabetes has been treated by blocking the
activating receptors on T cells with monoclonal
antibodies. In one such study, anti-lymphocyte serum
(ALS) and antibodies directed against CD4 and Cd8 T cell
25   receptors were administered to diabetic mice. T. Maki
et al., Long-term Abrogation of Autoimmune Diabetes in
Non-obese Diabetic Mice by Immunotherapy with Anti-
lymphocyte Serum, 89 Proc. Nat'l Acad. Sci. USA 3434-38
(1992). In this study, ALS or anti-DC4 and CD8
30   treatment given within 14 days after disease onset
resulted in remission rates (euglycemia) of 75% and 65%,
respectively. Euglycemia occurred within 30 days after
treatment and lasted for about 200 days. Several
significant points about the autoimmunity of diabetes
35   were observed. The lymphocytic antibodies were
responsible for termination of the immune response,
thereby allowing islet recovery. Also, if antibody

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treatment was initiated prior to 14 days after disease
onset, there were sufficient numbers of viable islets
that could regenerate to resume normal glycemic
conditions. Unfortunately, this procedure may lead to
5   severely immune-compromised patients and may not be
useful for human applications. Similarly, other studies
demonstrate that the injection of anti-CD3 into NOD mice
can prevent diabetes.

In view of the foregoing, it will be appreciated
10 that providing a method of delaying the onset of IDDM in
an individual predisposed to this disease would be a
significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION
15 It is an object of the present invention to provide
a method for treating insulin dependent diabetes
mellitus (IDDM).

It is also an object of the invention to provide a
method for delaying the onset of IDDM in a person
20 predisposed to developing the disease.

It is another object of the invention to provide a
method for treating IDDM and delaying the onset thereof
by administering a modified anti-GAD antibody to a
person predisposed to developing the disease.

25 It is still another object of the invention to
provide a composition for administering to a person
predisposed to developing IDDM for delaying the onset of
IDDM.

These and other objects can be achieved by
30 providing a method for delaying onset of insulin
dependent diabetes mellitus in an individual predisposed
to developing the disease comprising administering to
the individual an effective amount of a composition
comprising (a) an immunologically active monoclonal
35 antibody or fragment thereof against glutamic acid
decarboxylase coupled to (b) a nonimmunogenic
hydrophilic polymer that provides a hydration shell

around the monoclonal antibody or fragment thereof for inhibiting immune recognition thereof.

Preferably, the polymer is a poly(ethylene glycol), and more preferably has a molecular weight in the range of about 200 to 8,000, although higher molecular weight polymers, branched polymers, star molecules, and PEG block copolymers are also within the scope of the invention. Methoxy-PEG is a particularly preferred polymer. It is also preferred that the monoclonal antibody or fragment thereof is an F(ab') fragment. Preferably, the monoclonal antibody or fragment thereof is against the GAD-65 isomer of glutamic acid decarboxylase. In one illustrative preferred embodiment, the monoclonal antibody and polymer are covalently coupled together with a crosslinker.

A method for reducing insulitis in beta cells of an individual predisposed to developing insulin dependent diabetes mellitus comprises administering to the individual an effective amount of a composition comprising (a) an immunologically active monoclonal antibody or fragment thereof against glutamic acid decarboxylase coupled to (b) a nonimmunogenic hydrophilic polymer that provides a hydration shell around the monoclonal antibody or fragment thereof for inhibiting immune recognition thereof.

A composition for delaying onset of insulin dependent diabetes mellitus in an individual predisposed to developing the disease comprises (a) an immunologically active monoclonal antibody or fragment thereof against glutamic acid decarboxylase coupled to (b) a nonimmunogenic hydrophilic polymer that provides a hydration shell around the monoclonal antibody or fragment thereof for inhibiting immune recognition thereof.

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BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 shows the reactions for coupling methoxy-PEG-amine to an F(ab′) fragment with a heterobifunctional crosslinker.

FIG. 2 shows the reactions for coupling diamino-PEG to an F(ab′) fragment with a heterobifunctional crosslinker and labeling the PEG moiety with label R, such as Bolton-Hunter reagent or fluorescein isothiocyanate.

FIG. 3 shows a plot of diabetic incidence as a function of time for NOD mice injected weekly with 0.3 mg anti-GAD Mab (●); 0.1 mg anti-GAD Mab (○); or not injected (□).

FIG. 4 shows a histogram of the percentage of islets exhibiting a relative islet insulitis score for NOD mice injected weekly with 0.3 mg anti-GAD Mab (hatched); 0.1 mg anti-GAD Mab (shaded); or not injected (unshaded).

FIG. 5 shows a histogram of in vitro splenocyte activation by anti-GAD Mab (unshaded) and polyclonal mouse IgG (shaded) at various Mab concentrations.

DETAILED DESCRIPTION

Before the present method for treating IDDM, and in particular delaying the onset thereof, is disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular

forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a composition comprising "an antibody" includes reference to two or more of such antibodies, and reference to "a polymer" includes reference to one or more of such polymers.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "effective amount" means an amount of the presently claimed composition to provide the selected effect and performance at a reasonable benefit/risk ratio attending any medical treatment. The guidance provided herein is sufficient to permit a person skilled in the art to determine appropriate dosages without undue experimentation.

As used herein, "administering" and similar terms mean delivering the composition to the individual being treated such that the composition is capable of being circulated systemically to the parts of the body where the antibody portion of the composition can bind its cognate antigen, e.g. islet cells. Thus, the composition is preferably administered to the individual by systemic administration, typically by subcutaneous, intramuscular, or intravenous administration, or intraperitoneal administration. Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Suitable excipients include, for example, water, saline, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances such as wetting or emulsifying agents, buffers, and the like can be added.

As used herein, "delaying onset" of IDDM and similar terms mean delaying the date wherein an individual predisposed to developing IDDM exhibits

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clinical symptoms of the disease, and include delaying
IDDM to the extent that the disease does not fully
develop.

An used herein, "poly(ethylene glycol)," "PEG," and
similar terms mean poly(ethylene glycol) and various
derivatives thereof, such as methoxy-PEG-amine, diamine-
PEG, and the like. Preferred poly(ethylene glycols)
include polymers of a molecular weight of about 200 to
8,000, but higher molecular weight polymers are also
within the scope of the invention. PEG includes linear
and branched polymers, star molecules, and PEG block
copolymers formed by the coupling of at least two
different PEG polymers to form a higher molecular weight
polymer.

In the present invention, anti-GAD monoclonal
antibodies (Mab) are modified to maintain binding to
their cognate antigens while further preventing
recognition by other aspects of the immune system. In
an illustrative embodiment, the anti-GAD antibody is
modified by digestion with a protease and chemical
reduction with a reducing agent to yield F(ab')
fragments, which are then conjugated with various
poly(ethylene glycol) polymers (PEG). The F(ab')
fragment retains the antigen-specific Fab binding
fragment, while the immune and complement activating Fc
fragment is removed. In addition, the poly(ethylene
glycol) moiety provides an increased hydration sphere
and dynamic mobility that prevents protein and cellular
interaction. Thus, the present anti-GAD-F(ab')-PEG
composition simultaneously binds GAD and prevents or
inhibits further recognition by the immune system.

Modern immunological techniques have made
biosynthesis, isolation, and purification of an antigen-
specific Mab a common laboratory procedure, E. Harlow &
D. Lane, Antibodies: A Laboratory Manual (1988), hereby
incorporated by reference. Animals can be immunized

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with a specific antigen to generate high plasma titers
of a selected antigen. Concurrently, primed B cells are
produced that synthesize the specific antibody. Primed
B cells (plasma cells) are then fused with a myeloma
5   cell line to produce large quantities of the antibody.
Through cell screening procedures, these hybridoma cells
can be selected for the specific Mab desired and will
produce the specific Mab either in cell culture or after
injection into a host animal.

10   As is well known in the art, antibodies of the IgG
class are divalent molecules. The so-called Fab portion
binds with the antigen, and the Fc portion can bind with
complement   proteins   or   with   other   immunity-related
cells,   such   as   T   cells   and   macrophages,   thereby
15   eliciting another antigenic-like component. However, an
intact   IgG   molecule   can   be   reproducibly   digested   by
papain to yield single Fab fragments or with pepsin to
obtain $F(ab')_2$ fragments, both of which lack the Fc
domain.   Digestion   with   pepsin   cleaves   the $F(ab')_2$
20   fragment   below   the   disulfide   hinge   region.   Thus,
chemically   reducing   the   disulfide   bonds   between   the
hinge region of the $F(ab')_2$ fragment yields two $F(ab')$
fragments, each with free, reactive sulfhydryl groups.
Since the antigen binding region is associated with the
25   V   (variable)   region   of   the $F(ab)$ domain, $F(ab')$
fragments   maintain   the   same   antigen   binding   properties
as the intact IgG molecule.   Such $F(ab')$ fragments are
advantageous   because   of   the   availability   of   free
sulfhydryl groups for subsequent formation of covalent
30   chemical   linkages   with   fluorescent   labels,   anticancer
drugs, polymers, and the like.

                Furthermore, Mab's and fragments thereof have been
used in targeted drug delivery.   P. Tyler & B.P. Ram,
Monoclonal Antibodies, Immunoconjugates and Lipsomes as
35   Targeted Therapeutic Systems, in P. Tyle & B.P. Ram
eds.,   Targeted   Therapeutic   Systems   3-24   (1990).
Antibodies   administered   to   experimental   animals   and

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humans have been targeted to cell surface antigens
presented on the tumor cells. The Mab's can destroy
tumor cells through inhibition of these surface
receptors involved in metabolic activity, or through
5   signaling events. Increased therapeutic activity of
drugs against tumors has been achieved through coupling
cytotoxic drugs with Mab's, thus directing the agent
directly to the tumor mass. S. Ramakrishnan, Current
Status of Antibody-toxinconjugates for Tumor Therapy, in
10   P. Tyle & B.P. Ram eds., Targeted Therapeutic Systems
189-215 (1990). In addition, liposomes bound with Mab
F(ab') fragments have also been used in antitumor
treatment. The liposomes act as a drug reservoir, which
can be deposited and accumulated in the vicinity of the
15   tumor mass through the targeting or homing properties of
the Mab/F(ab') fragment. Therefore, Mab's can be
administered to an individual such that the Mab's bind
to and accumulate on a selected target antigen.

Mab's against the specific beta cell antigen, GAD,
20   can be prepared according to methods well known in the
art. G. Kohler & C. Milstein, Continuous Cultures of
Fused Cells Secreting Antibody of Pre-defined
Specificity, 256 Nature 495-97 (1975);
Wunderlich et al., 17 Eur. J. Cancer Clin. Oncol. 719
25   (1981); Schlom et al., 77 Proc. Nat'l Acad. Sci. USA
6841 (1980); E. Harlow & D. Lane, Antibodies: A
Laboratory Manual (1988), hereby incorporated by
reference. A hybridoma cell line generating anti-GAD-
IgG is also available from the ATCC (accession no.
30   HB184).

The Mab generated against GAD is digested to obtain
the F(ab') fragment, according to methods well known in
the art. J. Rousseaux et al., Optimal Conditions for
the Preparation of Proteolytic Fragments from Monoclonal
35   IgG of Different Rat IgG Subclasses, 121 Meth. Enzymol.
663-69 (1986); S.I. Wie et al., Characterization of the
Proteolytic Fragments of Bovine Colostral IgG[1], 121 J.

Immunol. 98-104 (1978), hereby incorporated by
reference. To further increase the immune reactivity of
the F(ab') fragment, poly(ethylene glycol) (PEG) is
conjugated to the F(ab') molecule. PEG is a linear or
5   branched, neutral polyether, available in a broad range
of molecular weights, and is soluble in water and most
organic solvents. PEG is effective at excluding other
polymers or peptides from its presence when in water,
primarily through its high dynamic chain mobility and
10   hydrophilic nature, thus creating a water shell or
hydration sphere when attached to other proteins or
polymer surfaces. PEG is nontoxic, non-immunogenic, and
approved by the Food and Drug Administration for
internal consumption.

15   PEG conjugated polyers, proteins, or enzymes have
demonstrated bioactivity, non-antigenic properties, and
decreased clearance rates when administered in animals.
F.M. Veronese et al., Preparation and Properties of
Monomethoxypoly(ethylene glycol)-modified Enzymes for
20   Therapeutic Applications, in J.M. Harris ed.,
Poly(Ethylene Glycol) Chemistry--Biotechnical and
Biomedical Applications 127-36 (1992). This is due to
the exclusion properties of PEG in preventing
recognition by the immune system. In addition, PEG has
25   been widely used in surface modification procedures to
decrease protein adsorption and improve blood
compatibility. S.W. Kim et al., Nonthrombogenic
Bioactiev Surfaces, 516 Ann. N.Y. Acad. Sci. 116-30
(1987); H. Jacobs et al., Surface Modification for
30   Improved Blood Compatibilty, 12 Artif. Organs 500-01
(1988); K.D. Park et al., Synthesis and Characterization
of SPUU-PEO-Heparin Graft Copolymers, 29 J. Poly. Sci,
Part A 1725-31 (1991). Hydrophobic polymer surfaces,
such as polyurethanes and polystyrene were modified by
35   the grafing of PEG (MW 3,400) and employed as
nonthrombogenic surfaces. In these studies, surface
properties (contact angle) were more consistent with

hydrophilic surfaces, due to the hydrating effect of PEG. More importantly, protein (albumin and other plasma proteins) adsorption was greatly reduced, resulting from the high chain motility, hydration sphere, and protein exclusion properties of PEG.

PEG MW 3,400 was determined as an optimal size in surface immobiliztion studies, K.D. Park et al., Blood Compatibility of SPUU-PEO-Heparin Graft Copolymers, 26 J. Biomed. Mat. Res. 739-45 (1992), while PEG MW 5,000 was most beneficial in decreasing protein antigenicity, F.M. Veronese et al., in J.M. Harris ed., Poly(Ethylene Glycol) Chemistry--Biotechnical and Biomedical Applications 127-36, supra.

                            Example 1
Purification of Antibodies from Cell Culture

        A hybridoma cell line producing anti-GAD-IgG (IgG$_1$), ATCC no. HB184, was stored frozen (-20°C). An aliquot of frozen cells was thawed and suspended in a tissue culture flask containing 90% Dulbecco's modified Eagle medium with 4.5 g/l glucose and 10% fetal bovine serum (Hyclone, Logan, Utah). The medium was renewed every 2-3 days by dilution with fresh medium to maintain a cell density between 10^5 and 10^6 cells/ml. The colony was maintained at 37°C under 6.5% CO_2 atmosphere.

        The ability of the cell suspension to secrete anti-GAD-IgG was continually monitored using an enzyme linked immunosorbent assay (ELISA). In this assay, GAD (Sigma Chemical Co., St. Louis, MO) was dissolved in a coating buffer (50 mM sodium phosphate, 1.2 M NaCl, pH 7.5) and the solution (0.5 mg/ml) was transferred to wells of a microplate (50 µl/well) and incubated for 1 hour at 37 °C. The wells were washed with 200 µl of blocking buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, 0.02% Tween 20, 1% bovine serum albumin, pH 7.3) and then incubated in 250 µl of blocking buffer for 1 hour at 37°C. After incubation, the blocking buffer was removed and the

microplate was dried. Duplicate dilutions (50 μl) of
samples containing anti-GAD (IgG, F(ab'), or F(ab')-PEG)
(serum or dilutions of chromatographic fractions) were
placed in the wells and incubated for 2 hours at 37°C,
5   followed by 3 washes with washing buffer (blocking
buffer without BSA). Rabbit anti-IgG$_1$ mouse
immunoglobulin conjugated with alkaline phosphatase, at
a concentration of 1/200 in blocking buffer, was added
to all wells and incubated for 2 hours at 37°C, followed
10   by 3 washes with washing buffer. The wells were then
incubated with p-nitrophenol phosphate chromogen at 1
mg/ml in 1 M diethylethanolamine buffer for 40 minutes
at room temperature. The optical density at 405 nm was
then determined using a microplate autoreader (EL311,
15   Bio-Tek Instruments). These values were compared to
standard curves prepared with known anti-GAD
concentrations to extrapolate the anti-GAD antibody
concentration.

The IgG-containing cell culture medium was isolated
20   by centrifuging the cell suspension for 10 minutes at
1400 g and discarding the cell pellet. The supernatant
containing anti-GAD-IgG was then concentrated 10 to 15
times against a sodium phosphate buffer with a
concentrator (Centriprep, MWCO = 100,000).

25   The concentrated medium was then filtered through
a 0.2 μm filter (Sterile Acrodisc, Gelman Sciences) and
loaded onto a DEAE-cellulose column, previously
equilibrated with 25 mM sodium phosphate pH 7.3. The
bound proteins were eluted with PBS buffer at pH 7.33
30   (40 mM dibasic sodium phosphate, 10 mM monobasic sodium
phosphate, 1.5 M NaCl) at a flow rate of 1 ml/minute.
After each run, the column was rinsed with 2 volumes of
0.1 M HCl, followed by 2 volumes of 0.1 M NaOH, and
reequilibrated with 25 mM sodium phosphate, pH 7.3.
35   Typically, antibodies were eluted in the non-retained
fraction. The purity of the fraction was characterized
by electrophoresis (Phastgel 8/25, Pharmacia) under

native and denaturing conditions. The activity of the
anti-GAD-IgG fraction was estimated by ELISA as
described above. This procedure typically resulted in
a yield of about 70-80% of the initial anti-GAD-IgG
concentration.

Example 2
Purification of Antibodies from Ascites Fluid

Ascites is an intraperitoneal fluid extracted from
mice with peritoneal tumors. Ascites fluid results from
hybridoma cells being injected intraperitoneally into
mice for growth of the hybridoma cells and Mab
production. The hybridoma cells grow to high densities
in the peritoneal cavity and continually secrete
antibodies specific to the hybridoma cell line.
Typically, antibody production by this method yields
approximately 3 ml of ascites fluid per mouse,
containing between 5 and 10 mg/ml of IgG, representing
between 90-98% of the crude antibody titer.

Retired Breeders BALB/c mice were purchased from
Jackson Laboratory. The mice were primed with pristane,
which acts as an intraperitoneal irritant to recruit
nutrients, monocytes, and lymphoid cells into the
peritoneum, thereby creating a favorable growth
environment for the hybridoma cells. The mice were then
injected with hybridoma cells (ATCC no. HB184) based on
the following timetable. Day 0 is the end of the
isolation period, upon which the mice were each injected
intraperitoneally with 0.5 ml of pristane. On day 3,
each mouse was again injected with pristane as before.
On day 10, each mouse was injected intraperitonally with
0.5 ml of hybridoma cell suspension (ATCC no. HB184)
containing 10^6 cells per ml. Between days 20 and 25, the
mice were euthanized by carbon dioxide asphyxiation, and
the ascites fluid was extracted from the intraperitoneal
cavity. After about 25 days, the animals were
euthanized with CO_2 gas, and the ascites fluid was

extracted from the peritoneal cavity. The fluid was centrifuged and the pellet containing the red cells and the fibrin clot was discarded. The ascites fluid was then stored at -20°C before purification.

5   The anti-GAD-IgG from the ascites fluid was purified by precipitation with saturated ammonium sulfate (SAS), followed by anion exchange chromatography and protein A-SEPHAROSE chromatography. The ascites fluid from 10 mice was slowly added to an equal volue of
10   SAS at 4°C under agitation. The solution was centrifuged and the supernatant was separated from the precipitate, both being retained. The pellets were redissolved in PBS and precipitated again with an equal volume of SAS, the supernatant was reprecipitated with
15   0.5 volume of SAS, and then the two mixtures were incubated overnight at 4°C. After incubation, the two solutions were centrifuged, the supernatant of each was discarded, and the pellets were redissolved in a volume of PBS corresponding to 0.25 of the initial volume of
20   ascites fluid. The solutions were dialyzed overnight at 4°C, filtered through a 0.2 μm filter, and eluted on a DEAE-cellulose column, as described in Example 1.

      Depending on the purity, a second chromatographic purification on a protein A-SEPHAROSE column may be
25   necessary. The Mab-containing fraction was diluted with an equal volume of binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9). The bound protein was eluted from the protein A column with 0.1 M sodium citrate, pH 6.0, and subsequently with 0.1 M sodium citrate, pH 3.0. After
30   each purification, the column was rinsed with two volumes of 6 M guanidine. Typically, the anti-GAD antibody emerged as a sharp peak eluted in 0.1 M sodium citrate, pH 6.0. The yield of the anti-GAD-IgG from ascites fluid was typically 30-40%.

35   It was anticipated that the antibodies isolated during the previous procedures were a mixture of anti-GAD and indigenous mouse antibodies. As a final

purification step, a GAD-immobilized affinity column was
prepared that binds only anti-GAD-IgG. CDI-activated
SEPHAROSE beads (Pierce Chemical Co.) were covalently
bound to GAD through ϵ-amino groups of lysine residues.
5   The beads were prepared and the GAD coupled to the beads
according to the manufacturer's specifications. The Mab
preparation was dissolved in 0.1 M borate buffer (pH
8.5) and added to the CDI-activated bead slurry (about
2 mg GAD/ml of beads) and gently mixed overnight at 4°C.
10   The beads were filtered and washed with 2 M Tris (pH
8.0) to block remaining CDI groups. The Mab solution in
PBS was then bound to the beads packed in a column. The
column was washed with 10 volumes of PBS to remove
unbound IgG. Anti-GAD-IgG was displaced and eluted by
15   an ionic strength gradient. The purified fractions were
pooled, dialyzed, lyophilized, and stored at -20°C.

                          Example 3
       Preparation of Anti-GAD-F(ab') Fragments
20   The anti-GAD-IgG prepared according to the
procedures of either Example 1 or Example 2 was
enzymatically digested and then chemically reduced to
obtain F(ab') fragments, which could then be coupled to
PEG. The rationale behind this procedure is to obtain
25   an antibody fragment capable of binding to the GAD
antigen yet which lacks the Fc domain, and is conjugated
with PEG to further decrease protein and cellular
interactions.

          Digestion of intact IgG to yield F(ab')$_2$ domains is
30   a common laboratory procedure.   Anti-GAD-IgG was
dissolved in PBS at a concentration of 1-2 mg/ml, and
then 1 ml of 100 mM sodium citrate (pH 4.2) was added.
The pH of the solution was maintained at pH 4.2 with the
addition of 0.1 M acetic acid.   Pepsin (1 mg/ml),
35   corresponding to a ratio of 6 mg pepsin per 33 mg anti-
GAD-IgG, was added to the Mab solution and incubated for
12 hours at 37°C. A person of ordinary skill in the art

can modify the incubation time and ratio of pepsin to antibody, if necessary, to obtain an optimal yield of F(ab')$_2$ fragments. The digestion reaction was terminated by adjusting the pH of the solution to pH 6.5 with 0.1

5   M carbonate buffer (pH 9.5). The reaction mixture was then immediately loaded onto a size exclusion chromatography column (HILOAD 16/60, Pharmacia).

Additional purification of the F(ab')$_2$ fragments was performed on a protein A column. The Fc fragments and

10   intact IgG molecules bind to protein A, whereas the F(ab')$_2$ fragments do not. The F(ab')$_2$ peak was identified by UV spectroscopy at 280 nM, and the F(ab')$_2$ fractions were collected, lyophilized, and stored at - 20°C.

15   The disulfide bonds joining the two heavy chain fragments of the F(ab')$_2$ were reduced with dithiothreitol (DTT) to yield two F(ab') fragments per F(ab')$_2$ fragment, each with free, reactive sulfhydryl groups. An aliquot of F(ab')$_2$ fragments (0.67 ml at 3 mg/ml) was added to

20   0.23 ml of buffer (100 mM sodium acetate, 88 mM NaCl, pH 5.5) and 0.1 ml of 200 mM DTT, and then was gently mixed at room temperature for 90 minutes.

Separation of unreacted F(ab')$_2$ fragments and reagents was perfomed on a PD10 GPC column using 100 mM

25   sodium acetate, 88 mM NaCl, pH 5.5, as the elution buffer. Chromatography was performed in a nitrogen atmosphere (in a glove box) with all reagents and buffers thoroughly degassed and flushed with nitrogen. The elution volume containing the F(ab') fragments was

30   determined by UV spectroscopy at 280 nm.

Example 4

Activation of Methoxy-PEG-amine

As discussed previously, poly(ethylene glycols)

35   (PEGs) with molecular weights between about 2,000 and 8,000 have been used to prevent plasma protein adhesion on blood contacting surfaces and to decrease the

antigenicity of foreign immunogenic proteins and enzymes. Therefore, PEGs of various molecular weights are coupled to the F(ab') fragments through the sulfhydryl groups thereof. These anti-GAD-F(ab')-PEG compositions maintain ability to bind to islet/beta cells while the PEG moiety masks the remainder of the F(ab') molecule from eliciting additional immunological events.

PEG coupling to F(ab') fragments can use a heterobifunctional crosslinking agent, such as sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (sulfo-SMCC; Pierce Chemical Co.). This agent is first coupled to an amine group of methoxy-PEG-amine $(CH_3-[O-CH_2-CH_2]_n-NH_2)$ and then is linked to the F(ab') fragment by the sulfydryl group thereof (FIG. 1).

Methoxy-PEG-amine, with a molecular weight ranging from about 200 to 8,000 (2×10^{-4} moles) is dissolved in 0.1 sodium phosphate, pH 9.0. A 0.5 molar equivalent of sulfo-SMCC (0.044 mg, 1×10^{-4} moles) is added and the reaction proceeds for 24 hours at 4°C.

A cationic exchange column is used to separate the reaction mixture into substituted and unsubstituted SMCC-PEG derivatives. The reaction mixture containing 100 mg of PEG derivatives is applied to a MONO S chromatography column (Pharmacia) connected in line with a fast protein liquid chromatography (FPLC; Pharmacia) system. A linear gradient of NaCl is used to elute the PEG derivatives, wherein the substituted and unsubstituted derivatives elute at different ionic strengths. The eluate is monitored with a UV detector set at the maximum absorbance of the maleimide group. The peak corresponding to the pure substituted SMCC-PEG is collected, dialyzed against distilled water, lyophilized, and stored at -20°C.

Example 5

Activation of Diamino-PEG

For cell staining and whole body perfusion
(pharmacokinetic) evaluations, it is useful to label
5   anti-GAD-F(ab')-PEG with, for example, a radioactive or
fluorescent label. In vivo therapeutic applications of
the anti-GAD-F(ab')-PEG generally do not require such
labels. Current methods of labeling antibodies involve
forming conjugates through amine groups (fluorescent or
10   ^{125}I labels) or through oxidation of tyrosine residues
(^{125}I label). These labeling methods can interfere with
antibody binding through reaction with the active site
of the antibody. Therefore, this example shows coupling
of the label to the PEG moiety. The labeled PEG moiety
15   is later coupled to the F(ab') fragment. This procedure
assures that labeled and unlabeled compositions have
similar affinities for the antigen.

The procedure of Example 4 is followed with the
exception that diamino-PEG ($H_2N-[O-CH_2-CH_2]_n-NH_2$) is used
20   instead of methoxy-PEG-amine. The aim is to modify one
amine group of the diamino-PEG while leaving the other
amine group available for reaction with a label (FIG.
2). The reaction of diamino-PEG with sulfo-SMCC results
in a mixture of di-, mono-, and un-substituted SMCC-PEG
25   derivatives. This mixture is separated according to the
procedure of Example 4. Elution with low ionic strength
buffer results in elution of the disubstituted SMCC-PEG
derivatives (both amine groups coupled, no ionic
binding). Monosubstituted and unsubstituted PEGs elute
30   at higher ionic strengths. The pure monosubstituted
SMCC-PEG is collected, dialyzed against distilled water,
lyophilized, and stored at -20°C.

Example 6

35   ^{125}I Labeling of Activated PEG

In this example, the monosubstituted SMCC-PEG-amine
of Example 5 is labeled by iodination. The SMCC-PEG-

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amine (0.5 g) is dissolved in 0.1 M sodium borate (pH 8.5) to a final concentration of 0.1 mg/ml and transferred to an ice bath. About 1 mCi of ^{125}I-labeled Bolton-Hunter reagent is added to a 1.5 ml tube at 0°C, and the solvent is evaporated with nitrogen gas. The SMCC-PEG-amine solution is added to the dried Bolton-Hunter reagent and permitted to react for 15 minutes on ice. An equal volume of "stop" solution (0.5 M ethanolamine, 10% glycerol, 0.1% xylene cyanol in 0.1 M sodium phosphate, pH 8.5) is added and incubated for 5 minutes at room temperature. Purification will be accomplished by dialysis (MWCO 3,500) against distilled water. The purified product is then lyophilized and stored at -20°C.

Example 7
Fluorescein Labeling of Activated PEG

In this example, the monosubstituted SMCC-PEG-amine of Example 5 is labeled with fluorescein isothiocyanate (FITC). SMCC-PEG-amine (2 mg/ml) is dissolved in 0.1 M sodium carbonate (pH 9.0). FITC is dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. FITC is slowly added to the SMCC-PEG-amine solution with stirring until a concentration of 0.05 ml/mg of SMCC-PEG-amine is reached, and then the reaction is permitted to proceed in the dark for 8 hours at 4°C. A stop solution (50 mM ammonium chloride, 0.1% cylene cyanol, and 5% glycerol) is addd an incubated for 2 hours at 4°C. The resulting SMCC-PEG-FITC is purified and isolated by dialysis in the dark at 4°C against distilled water. The final compound is then lyophilized and stored at -20°C.

Example 8
Coupling of Anti-GAD-F(ab') to Activated PEG

In this example, a PEG intermediate prepared according to the procedure of Examples 4, 6, or 7 is

coupled to anti-GAD-F(ab') prepared according to the procedure of Example 3. All of the PEG intermediates described have maleimide groups for coupling to the sulfhydryl moieties of F(ab') fragments. The purified anti-GAD-F(ab') fragments are added to solutions of the selected PEG intermediate in a molar ratio of 3 PEG intermediates to 2 F(ab') fragments in 100 mM sodium acetate, 88 mM NaCl, pH 5.5. The reaction proceeds in a nitrogen atmosphere for 24 hours at 4°C.

Purification of the anti-GAD-F(ab')-PEG is accomplished by equilibrium dialysis at 4°C. Appropriate precautions are taken for FITC-labeled (dark) and radioactively-labeled compositions. The reactions products are placed in cellulose acetate dialysis bags (MWCO <14,000) and dialyzed against PBS for 48 hours with frequent changes of buffer. The higher molecular weight anti-GAD-F(ab')-PEG is retained in the bag, while unreacted PEG intermediates are removed.

The protein content of the purified composition is determined by standard protein assay, e.g., M. Bradford, A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding, 72 Anal. Biochem. 248-54 (1976). Labeled compositions are assayed by, for example, fluorescence for FITC-labeled compositions or gamma counting for [125]-labeled compositions.

Example 9
In Vivo Treatment of NOD Mice with Anti-GAD Mab

The non-obese diabetic (NOD) mouse is an excellent model to study Type I diabetes in relation to humans. H. Ikegami et al., Immunogenetics and Immunopathogenesis of the NOD Mouse, in G.S. Eisenbarth ed., Immunotherapy of Diabetes and Selected Autoimmune Diseases 24-31 (1980); S. Makino et al., Breeding of a Non-obese, Diabetic Strain of Mice, 12 Exp. Anim. 1-15 (1980). NOD

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mice spontaneously develop diabetes within 16 weeks of
birth and demonstrate cell pathology and organ system
failure similar to humans. Generally, overt diabetes
begins at about 12 weeks of age and is observed in
5   nearly 90% of females and 20% of males within 16 weeks.
The animals display preclinical antibody titers,
insulitis within 4-6 weeks, and cell infiltration
(macrophage, T cell, and B cell) prior to beta cell
destruction, as observed in humans.   They develop
10   clinical symptoms such as polyuria, glycosuria, and loss
of weight, as well as hyperglycemia, increased
hemoglobin A1 (glycosylated hemoglobin), and deficit of
intrinsic insulin production. Extensive studies on gene
manipulation, immunotherapy, and lymphocyte inhibition
15   have established the similarity of IDDM between NOD mice
and humans. Therefore, the NOD mouse is an animal model
viewed by one skilled in the art as being reasonably
predictive of utility of a treatment for IDDM in humans.
In this example, a group of NOD mice was
20   administered anti-GAD Mab prepared by the procedure of
Example 2 by weekly intraperitoneal injection with
either 0.1 or 0.3 mg of the Mab beginning at 7 weeks of
age.   Controls were not injected. The animals were
weighed and their blood glucose levels determined
25   weekly. Blood glucose levels were determined by cutting
the tail and collection a blood sample (about 150 μl) in
a capillary tube. A portion of the blood was placed on
commercial glucose test strips and the blood glucose
levels determined. Prior to experimental evaluation,
30   hyperglycemic animals received 0.3-0.5 IU of 50:50
regular/NPH insulin twice daily.   Animals were
considered to be diabetic with a blood glucose level of
>200 mg/dl. FIG. 3 shows that the mice injected with
anti-GAD Mab exhibited a substantial reduction in
35   disease incidence, and those animals that did develop
diabetes did so at a substantially later date than
controls. These results show that administration of

anti-GAD antibodies can both reduce disease incidence and delay the onset of IDDM.

Example 10

5   In this example, the procedure of Example 9 is followed except that the NOD mice are injected with anti-GAD-F(ab')-PEG prepared according to the procedure of Example 8.

10   Example 11

In this example, euglycemic NOD mice were injected with anti-GAD Mab at doses of 0.3 mg and 0.1 mg (5 to 6 mice/group) according to the procedure of Example 9. The mice were sacrificed at 32 weeks of age and control 15   (uninjected) mice were sacrificed at about 20 weeks of age, suffering from hyperglycemia. The pancreata were removed, fixed with 10% buffered formalin, embedded in parafin, sectioned at 5 μm, and stained with hematoxylin and eosin. The severity of insulitis was assessed using 20   the following scoring system: (0) normal islet; (1) mononuclear infiltration in less than 25% of the islet; (2) 25 to 50% of the islets infiltrated; (3) over 50% infiltrated; (4) extensive intra-islet infiltration with obvious beta cell damage. The mean score for each 25   pancreas was calculated by dividing the total score by the number of islets scored (FIG. 4).

These pancreas histology studies show that the anti-GAD Mab treated mice have a lower level of insulitis as compared to the control group by 16 weeks.
30

                          Example 12

In this example, the procedure of Example 11 is repeated with the exception that anti-GAD-F(ab')-PEG prepared according to the procedure of Example 8.
35

Example 13

In this example, 8-week-old NOD mice were sacrificed and their spleens were removed aseptically and placed in ice-cold RPMI 1640 medium. Splenocytes were extracted by disrupting the spleens in a solution containing 0.15 M Tris, 0.83% NH_4Cl, and then passing the cells through a fine wire mesh. Splenocytes (5 x 10^6 cells/well) were cultured for 4 days in different concentrations of anti-GAD Mab (0 to 10 μg/well) or polyclonal mouse IgG (0 to 10 μg/well; Zymed). The extent of cell proliferation in each cell was assayed using a colorimetric technique using microtetrazolium (MTT). Thus, MTT was dissolved in sterile PBS, pH 7.3, at a concentration of 5 mg/ml and then filter sterilized. This solution (25 μl) was added to each well. Incubation was continued for 4 hours, after which 100 μl of a solubilizing solution (20% (w/v) SDS, 50% dimethylformamide, 50% H_2O, adjusted to pH 4.7 by addition of acetic acid and HCl) was added to each well, and incubation was continued for 4 additional hours. The absorbance of each well was then read at 570 nm. MTT reacts with an enzyme in metabolically active cells such that it is converted to a product that absorbs at 570 nm. Thus, the absorbance measured in each well is proportional to the number of viable cells per well.

FIG. 5 shows the results of these experiments demonstrating the effects of the anti-GAD Mab on splenocyte cells. The incidence of splenocyte activation by anti-GAD Mab demonstrated a direct effect of anti-GAD Mab on lymphocyte activation. This indicates that a specific regulation of the idiotypic network by anti-GAD Mab can induce the regulation of the immune system and prevent or inhibit diabetes in NOD mice. This activation appears to be concentration dependent in that there was little or no effect observed at low concentrations (0-1 μg/well), but there was significant stimulation of cell proliferation at higher

concentrations (10 μg/well) of anti-GAD Mab.   There was
no significant proliferation observed in the polyclonal
mouse   IgG   treated   control   cells   under   the   same
conditions.

5

                              Example 14
         In this example, the procedure of Example 13 is
followed with the exception that anti-GAD-F(ab')-PEG
prepared according to the procedure of Example 8 is
10   substituted for anti-GAD Mab.

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CLAIMS

We claim:

1.   A method for delaying onset of insulin
dependent diabetes mellitus in an individual predisposed
5   to developing the disease comprising administering to
said individual an effective amount of a composition
comprising (a) an immunologically active monoclonal
antibody or fragment thereof against glutamic acid
decarboxylase coupled to (b) a nonimmunogenic
10   hydrophilic polymer that provides a hydration shell
around said monoclonal antibody for inhibiting immune
recognition thereof.

2.   The method of claim 1 wherein said polymer is
15   a poly(ethylene glycol).

3.   The method of claim 2 wherein said
poly(ethylene glycol) has a molecular weight in the
range of about 200 to 8,000.
20

4.   The method of claim 2 wherein said monoclonal
antibody or fragment thereof is an F(ab') fragment.

5.   The method of claim 2 wherein said
25   poly(ethylene glycol) is methoxy-poly(ethylene glycol).

6.   The method of claim 5 wherein said monoclonal
antibody or fragment thereof is an F(ab') fragment.

30   7.   The method of claim 2 wherein said monoclonal
antibody or fragment thereof is covalently coupled to
said poly(ethylene glycol).

8.   The method of claim 7 wherein said monoclonal
35   antibody or fragment thereof is covalently coupled to
said poly(ethylene glycol) with a crosslinker.

9.   The method of claim 8 wherein said crosslinker is sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate).

5   10.   The method of claim 9 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

11.   The method of claim 1 wherein said monoclonal antibody or fragment thereof is against a GAD-65 isomer
10   of glutamic acid decarboxylase.

12.   The method of claim 11 wherein the monoclonal antibody or fragment thereof is derived from ATCC no. HB184.
15

13.   A method for reducing insulitis in beta cells of an individual predisposed to developing insulin dependent diabetes mellitus comprising administering to said individual an effective amount of a composition
20   comprising (a) an immunologically active monoclonal antibody or fragment thereof against glutamic acid decarboxylase coupled to (b) a nonimmunogenic hydrophilic polymer that provides a hydration shell around said monoclonal antibody for inhibiting immune
25   recognition thereof.

14.   The method of claim 13 wherein said polymer is a poly(ethylene glycol).

30   15.   The method of claim 14 wherein said poly(ethylene glycol) has a molecular weight in the range of about 200 to 8,000.

16.   The method of claim 14 wherein said monoclonal
35   antibody or fragment thereof is an F(ab') fragment.

17. The method of claim 14 wherein said poly(ethylene glycol) is methoxy-poly(ethylene glycol).

18. The method of claim 17 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

19. The method of claim 14 wherein said monoclonal antibody or fragment thereof is covalently coupled to said poly(ethylene glycol).

20. The method of claim 19 wherein said monoclonal antibody or fragment thereof is covalently coupled to said poly(ethylene glycol) with a crosslinker.

21. The method of claim 20 wherein said crosslinker is   sulfosuccinimidyl   4-(N-maleimidomethyl)cyclohexane-1-carboxylate).

22. The method of claim 21 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

23. The method of claim 13 wherein said monoclonal antibody or fragment thereof is against a GAD-65 isomer of glutamic acid decarboxylase.

24. The method of claim 23 wherein the monoclonal antibody or fragment thereof is derived from ATCC no. HB184.

25. A composition for delaying onset of insulin dependent diabetes mellitus in an individual predisposed to   developing   the   disease   comprising   (a)   an immunologically active monoclonal antibody or fragment thereof against glutamic acid decarboxylase coupled to (b) a nonimmunogenic hydrophilic polymer that provides a hydration shell around said monoclonal antibody for inhibiting immune recognition thereof.

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26. The composition of claim 25 wherein said polymer is a poly(ethylene glycol).

27. The composition of claim 26 wherein said poly(ethylene glycol) has a molecular weight in the range of about 200 to 8,000.

28. The composition of claim 26 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

29. The composition of claim 26 wherein said poly(ethylene glycol) is methoxy-poly(ethylene glycol).

30. The composition of claim 29 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

31. The composition of claim 26 wherein said monoclonal antibody or fragment thereof is covalently coupled to said poly(ethylene glycol).

32. The composition of claim 31 wherein said monoclonal antibody or fragment thereof is covalently coupled to said poly(ethylene glycol) with a crosslinker.

33. The composition of claim 32 wherein said crosslinker is sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate).

34. The composition of claim 33 wherein said monoclonal antibody or fragment thereof is an F(ab') fragment.

35. The composition of claim 25 wherein said
monoclonal antibody or fragment thereof is against a
GAD-65 isomer of glutamic acid decarboxylase.

5   36. The composition of claim 35 wherein the
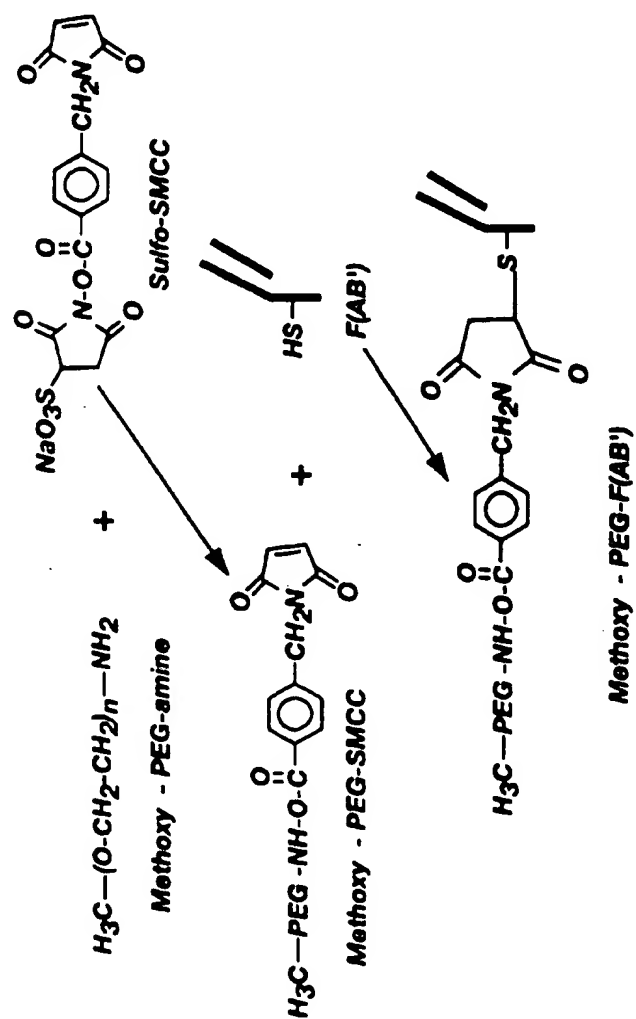monoclonal antibody or fragment thereof is derived from
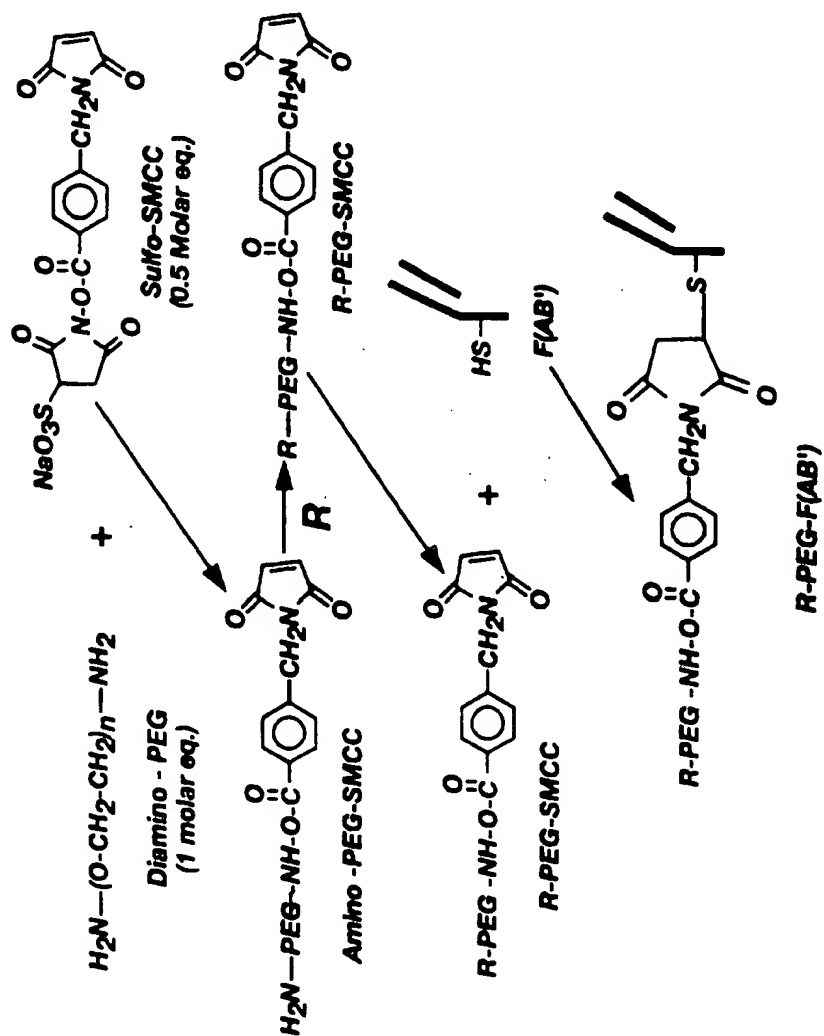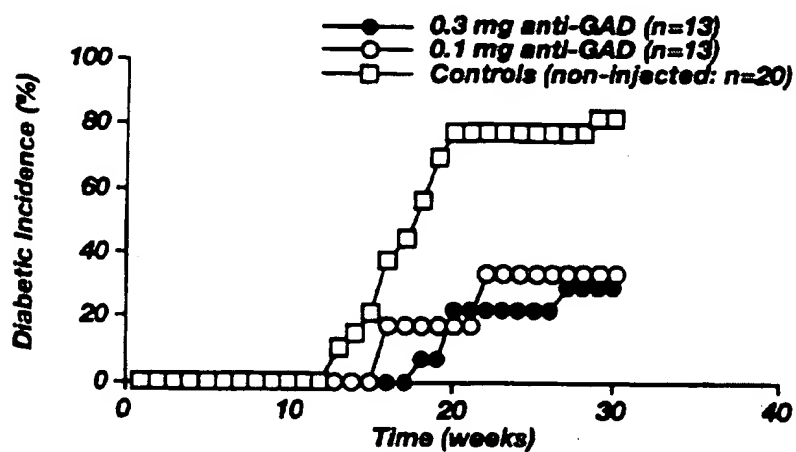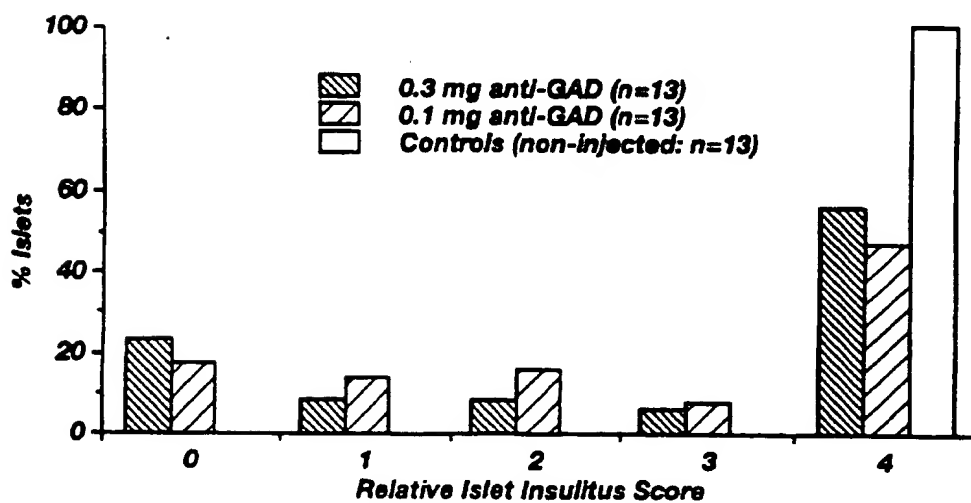ATCC no. HB184.

Fig. 1

Fig. 2

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Fig. 3

Fig. 4

Fig. 5

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A61K 39/44, 39/395; C07K 16/00
US CL   : 424/133.1; 530/387.1
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

   U.S. :   424/133.1; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

   APS, DIALOGUE. SEARCH TERM: antibodies against glutamic acid decarboxylase and treatment of diabetes

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAUFMAN, D.L. et al. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature. 04 November 1993, Vol 366, pages 69-71, see entire document.	1-36
Y	TISCH, R. et al. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature. 04 November 1993, Vol. 366, pages 72-75, see entire document.	1-36
Y	KABANOV, A.V. et al. A new class of drug carriers: micelles of poly(oxyethylene)-poly(oxypropylene) block copolymers as microcontainers for drug targeting from blood in brain. J. of Controlled Release. 1992, Vol 22, pages 141-157, see entire document.	1-36

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 DECEMBER 1996	30 JAN 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MINH-TAM B. DAVIS
Facsimile No (703) 305-3230	Telephone No. (703) 308-0916

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